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Feto-maternal immune regulation by TIM-3/galectin-9 pathway and PD-1 molecule in mice at day 14.5 of pregnancy

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ABSTRACT

Introduction: Immunoregulation implies the activation of negative pathways leading to the modulation of specific immune responses. Co-inhibitory receptors (such as PD-1 and TIM-3) represent possible tools for this purpose. PD-1 and TIM-3 have been demonstrated to be present on immune cells suggesting general involvement in immunosuppression such as fetomaternal tolerance. The aim of our study was to investigate the expression pattern of PD-1, TIM-3, and its ligand Gal-9 on different immune cell subsets in the peripheral blood and at the fetomaternal interface in pregnant mice.
Methods: TIM-3 and PD-1 expression by peripheral and decidual immune cells from pregnant BALB-c mice in 2 weeks of gestational age were measures by flow cytometry. Placental galectin-9 expression was determined by immunohistochemically and RT-PCR.
Results: Gal-9 was found to be present in the spongiotrophoblast layer of the haemochorial placenta. Decidual NK, NKT and γ/δ T cells showed increased PD-1 expression and reduced cytotoxic potential when compared to the periphery. TIM-3 expression by NK cells and γ/δ T cells is similar both in the periphery and in the decidua, notably, their relative TIM-3 expression is increased locally which is associated with reduced lytic activity. Decidual NKT cells exhibit a reduced TIM-3 expression with increased relative receptor expression and a slightly increased cytotoxicity when compared to the periphery.
Discussion: Our data reveals a particularly complex, tissue and cell type specific immunoregulatory mechanism by the investigated co-inhibitory receptors at the fetomaternal interface.

1. Introduction

During pregnancy, immunological recognition of the semi-allogenic fetus by the maternal immune system leads to a unique immune response phenomenon. At the very early stage of pregnancy, immunotolerance of the fetus has to be synchronized with decidual inflammatory processes required for successful implantation and placentation of the embryo. Later on, besides allowing fetal development the maternal immune system should be able to generate adequate responses to infections or eradicate nascent tumor cells. Therefore, controlling and regulating contradictory immune effector functions during pregnancy portrays a key role in maintaining immune homeostasis. Immunoregulation is mediated by a complex network of cellular and molecular interactions with the predominant role of regulatory T cells as cellular components of the immune system and an increased number of molecules with immunoregulatory capacity.

TIM-3 was first identified as a receptor specifically expressed by terminally differentiated IFN-γ producing CD4+ T helper 1 (Th1) and on CD8+ T cytotoxic cells [1]. Several studies demonstrated the expression of TIM-3 on a variety of immune cells including Th1, Th17, NK cells, NKT cells, CD4+CD25+ T cells (Tregs) and on cells of

Abbreviations: DC, dendritic cell; Gal-9, galectin-9; Gd, gestation day; PD-1, programmed cell death-1; RPMI 1640, Roswell Park Memorial Institute formulation 1640; QRT-PCR, quantitative real-time PCR; TIM-3, T cell immunoglobulion and mucin domain 3.

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the innate immune system, including dendritic cells (DCs), macrophages [2], and mast cells [3]. Mounting evidence supports the crucial role of TIM-3 in regulating immune responses [4] but the *in vivo* function of this receptor is still poorly understood. Using an allogeneic mouse model of pregnancy Chabtini et al. examined the TIM-3 expression on antigen presenting myeloid cells and indicated their possible role in the regulation of tolerance at the fetomaternal interface [5].

Galectin-9 (Gal-9), an endogenous lectin, was originally described as a potent eosinophil chemoattractant. Gal-9 can influence the immune system in different ways [6], either by exacerbating the inflammatory process [7] or by exhibiting therapeutic effects in autoimmune disease models, such as autoimmune arthritis, experimental allergic encephalomyelitis, and type 1 diabetes mellitus. Notably, it is generally believed that the engagement of Gal-9 by its receptor TIM-3 established the Gal-9/TIM-3 pathway as a novel regulator of Th1 immunity and tolerance induction [8].

TIM-3 is the most extensively studied Gal-9 receptor. A growing body of evidence proves that the engagement of these two molecules leads to the death of Th1 and Th17 cells thus negatively regulates IFN-γ secretion, furthermore influences the ability to induce T cell tolerance and triggers a significant signal cascade to induce apoptosis of Th1 type immune cells in both mice and humans [9–11]. Therefore, the connection between TIM-3 and Gal-9 may function as a negative regulator, dampening Th1- and Th17 driven immune responses and inducing peripheral tolerance by modulating the Th1/Th2 balance [12].

The TIM-3 mediated immunomodulation was first described as a clearly negative regulatory mechanism inducing immunotolerance and T cell apoptosis [13]. However, there are conflicting data surrounding the TIM-3 mediated regulation of innate immunity. In addition to suggesting similar immunosuppressive actions of TIM-3 on innate immune cells, as observed in T cells [14,15], TIM-3 was also found to promote innate immune responses [2,16]. Moreover, several recently published papers report that in reference to viral infections, overexpression of TIM-3 on NK cells was associated with effector dysfunction [17,18].

Programmed cell death-1 (PD-1) is a receptor and a member of the B7/CD28 family. It is known to downregulate T cell functions. PD-1 expression was observed on CD4+ Th- and CD8+ T lymphocytes, B lymphocytes, Tregs, NK, NKT cells, DCs, and activated monocytes [19]. PD-1 is not expressed on resting T cells but is inducible upon activation [20]. Known ligands of PD-1 include PD-L1 [21,22] and PD-L2 [23]. The binding of PD-1 on T cells with its ligand PD-L1 leads to decreased cytokine production and negatively regulates T cell proliferation and cell lysis during immune responses to pathogens or cancer [24]. Many publications revealed and characterized PD-1 as a classical exhaustion marker of T cells with poor effector functions [25,26].

Since pregnancy represents a unique model of local immunotolerance, regulatory pathways exerted by these co-inhibitory molecules could have significant impact on maternal immunosuppression. Therefore, the aim of our study was to investigate the expression pattern of TIM-3, PD-1 and Gal-9 on different immune cell subsets in the peripheral blood and at the fetomaternal interface.

2. Materials and methods

2.1. Animal model

Young (2 months) BALB-c mice were purchased from the Pécs Experimental Central Animal Laboratory and maintained on a 12 h light/dark cycle at 20–22 °C, 40–60% humidity, and were fed with standard feed pellets and tap water. Potential mates were paired up each evening and the presence of the copulatory plug was examined next morning. Once a plug is detected it is considered the gestation day (gd) 0.5. Gravid females were killed on gd 14.5 by cervical dislocation, the spleen and the uterine horns were aseptically removed. Animal housing, care, and application of experimental procedures were in accordance with institutional guidelines under approved protocols (No. BA02/2000–20/2006, University of Pecs).

2.2. Isolation of mononuclear cells from the spleen

Spleens were homogenized thoroughly with a syringe plunger, and single-cell suspensions were prepared using a 70-μm nylon cell strainer (BD Pharmingen). Subsequently, cells were washed in phosphate-buffered saline (PBS). Supernatant was aspirated and the pellet was resuspended in PBS and filtered via 40-μm nylon cell strainer (BD Pharmingen). Mononuclear cells were isolated on Ficoll–Paque (GE Healthcare) gradient. Cells were collected and resuspended in RPMI 1640 (Lonza) supplemented with penicillin (Lonza), streptomycin (Lonza) and 10% heat inactivated fetal calf serum (FCS) (Gibco).

2.3. Isolation of mononuclear cells from the decidua

The conceptus with the placentae was removed from the surrounding area of the endometrial tissue. Then the decidua was separated from the isolated placentae, sliced with scissors and finally digested with collagenase (Sigma–Aldrich) for 30 min at 37 °C. Placentas were removed and divided into pieces for RNA isolation followed by quantitative real-time PCR (qRT-PCR) and complete implantations were frozen for immunohistochemistry. The decidua next were homogenized thoroughly with a syringe plunger, and single-cell suspensions were prepared using a 70-μm nylon cell strainer. Subsequently, cells were washed in RPMI 1640 supplemented with penicillin, streptomycin and 10% FCS. The supernatant was aspirated and the pellet was resuspended in PBS and filtered via 40-μm nylon cell strainer. Mononuclear cells were isolated on Ficoll–Paque gradient. Cells were collected and resuspended in RPMI 1640.

2.4. Labeling of mononuclear cell and flow cytometric analysis

The isolated mononuclear cells (1×10^6 in 100 μl PBS/tube) were incubated for 30 min at room temperature (RT) with fluorochrome-labeled monoclonal antibodies. Following surface staining, the cells were washed with PBS, resuspended in 300 μl PBS containing 1% paraformaldehyde (PFA) and stored at 4 °C in the dark until fluorescence-activated cell sorting (FACS) analysis. Labeled cells were analyzed with a FACSCalibur flow cytometer (BD Immunocytometry Systems, Erembodegen, Belgium) equipped with the CellQuest software program (BD Biosciences, San Diego, CA, USA) and a BD FACSCanto II flow cytometer (BD Immunocytometry Systems, Erembodegen, Belgium) with the BD FACSDiva V6. software for data acquisition and analysis.

2.5. Antibodies

The following monoclonal antibodies were implemented:

Brilliant Violet (BV) 421-conjugated anti-mouse PD-1 (BD Pharmingen), BV510-conjugated anti-mouse CD3 (BD Pharmingen), BV510-conjugated anti-mouse γ/δ (BD Pharmingen), fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (BD Pharmingen), FITC-conjugated anti-mouse CD8 (BD Pharmingen), FITC-conjugated anti-mouse CD107a (BD Pharmingen), FITC-conjugated anti-mouse CD49b (BD Pharmingen), FITC-conjugated